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Missing pieces in protein deposition and mobilization inside legume seed storage vacuoles: calcium and magnesium ions

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Abstract

During the maturation of dicotyledonous seeds, organic carbon, nitrogen and sulphur are stored in protein storage vacuoles (PSVs) as storage globulins. Several studies point to the coexistence of storage proteins with proteases responsible for their degradation inside PSVs. Different mechanisms have been proposed to explain why there is no proteolysis during this period. Protein aggregation to form large supramolecular structures resistant to proteolytic attack could be the reason. However, during germination, and particularly following its completion, the globulin aggregates must undergo disintegration to allow protease attack for protein reserve mobilization. Based on the well-described concentration-dependent ability of Ca^{2+} and Mg^{2+} to promote *in vitro* aggregation and disaggregation of globulins, we explored a possible role for these alkaline earth cations in globulin packaging and mobilization. Ca^{2+} and Mg^{2+} measurements in purified PSVs [6.37 μmol and 43.9 μmol g^{-1} dry weight (DW) of cotyledons, respectively] showed the presence of these two alkaline earth cations within this compartment. To our knowledge, this is the first time that Ca^{2+} and Mg^{2+} have been quantified in purified PSVs from *Lupinus albus* seeds. Considering the importance of these two alkaline earth cations inside PSVs, which represent 14.6% and 60.7% of the total seed $Mg^{2+}$and Ca^{2+}, respectively, globulin aggregation and disaggregation profiles were assayed using experimental conditions closer to those that are physiologically present (proportion of Ca^{2+} and Mg^{2+}, and acidic pH). Based on: (1) the high *in vivo* abundance of Ca^{2+} and Mg^{2+} inside PSVs; and (2) globulin aggregation and disaggregation profiles, together with structural and physiological evidence already reported in the literature, an important physiological role for Ca^{2+} and Mg^{2+} in globulin packaging and mobilization inside PSVs is suggested.

Keywords: calcium, globulin aggregation–disaggregation, *Lupinus albus*, magnesium, protein storage vacuoles

Introduction

The turnover of seed storage proteins involves their synthesis during development and their degradation following germination. Globulins are the main storage proteins within the protein storage vacuoles (PSVs) of dicotyledonous seeds (Shewry and Casey, 1999), and several proteases responsible for their degradation are deposited along with them during seed development. However, degradation is negligible at this stage, indicating that storage proteins are protected against premature degradation (Shutov *et al.*, 2003; He *et al.*, 2007).

Several mechanisms have been proposed to explain how storage proteins avoid hydrolysis in developing seed PSVs. Jiang and Rogers (2002) suggested a differential sub-compartmentation of stored proteins and proteases, whereas several other authors proposed the maintenance of proteases in an inactive form (for reviews, see Muntz *et al.*, 2001; Tan-Wilson and Wilson, 2012). Protein structural changes were also proposed to prevent protease accessibility, as reported for the low specificity of papain-like proteases towards globulin crystals (Weber and Neumann, 1980). However, these structural alterations were not fully demonstrated. *In vitro* studies have shown that purified seed storage proteins from several legume species self-aggregate when incubated in the presence of Ca^{2+} and/or Mg^{2+}, whereas Cd^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and K^{+} are rather inefficient in promoting this (Ferreira *et al.*, 1999, 2003). The two alkaline earth cations, Ca^{2+} and Mg^{2+}, present in the cotyledons of legume seeds (Trugo *et al.*, 1993; Regvar *et al.*, 2011),

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may be considered promising candidates to fulfil an important physiological role in globulin self-aggregation *in vivo*, either by promoting efficient protein packaging in relatively small volumes and/or by conferring protection against proteolytic attack.

During germination, at the onset of protein storage mobilization, when there is no storage protein synthesis and when amino acids are mobilized to nourish the embryo, the mechanisms that protect stored proteins from degradation must be overcome. In dicotyledonous seeds, proteases stored in PSVs during maturation are apparently responsible for the initial protein mobilization, with the *de novo* synthesized proteases mediating the bulk of storage protein degradation only at a later stage (Muntz, 2007; Tan-Wilson and Wilson, 2012). The precise control of these proteolytic events during protein reserve deposition and mobilization remains largely unclear.

To help determine the physiological significance of alkaline earth cations on globulin mobilization, the *in vivo* concentrations of these elements were determined in both the cotyledons and PSVs isolated from *Lupinus albus* seeds. Globulin self-aggregation–disaggregation profiling, as a function of pH in the presence of those cations, was also evaluated. Selected protein structural data and physiological events reported in the literature were gathered and used to support a Ca^{2+}/Mg^{2+}-dependent model to explain how legume seed storage globulins are efficiently packed during seed formation, free from the action of neighbouring proteases, and subsequently dismantled and subjected to proteolytic digestion during seed germination and subsequent seedling growth.

Materials and methods

Plant material

Dry seeds of white lupin (*Lupinus albus* cv. Lublanc) were surface sterilised with 1% (v/v) sodium hypochlorite and germination was initiated by immersion of the seeds in running tap water for 2 d. The seed coats were removed and axes and intact cotyledons dissected from the embryos and stored at −80°C until required.

Protein purification

Lupinus albus seed globulins were purified from cotyledons of seeds germinated for 2 d, following a methodology similar to that described by Franco *et al.* (1997). Briefly, the cotyledons were ground in cold water (adjusted to pH 8.0) containing 0.01 M $CaCl_2$ and 0.01 M $MgCl_2$ [13 ml g^{-1} fresh weight (FW)] and stirred for 4 h. The suspension was filtered through a 20-μm mesh (Miracloth, CalBiochem, California, USA) prior to centrifugation for 1 h at 30,000 *g*. The resulting pellet was used for total globulin extraction by stirring it in a solution containing 10% (w/v) NaCl, 0.01 M EDTA and 0.01 M EGTA [13 ml $(g\ FW)^{-1}$], for 12 h. The suspension was centrifuged for 1 h at 30,000 *g* and the resulting globulin solution was concentrated by ammonium sulphate (561 g l^{-1}) precipitation. The precipitated globulins were centrifuged at 30,000 *g* for 20 min, resuspended in 0.02 M Tris-HCl at pH 7.5 and desalted on Econo-Pac 10 DG columns (BioRad, Hercules, California, USA), previously equilibrated in the same buffer. All operations were performed at 4°C.

Isolation of protein storage vacuoles

The protein storage vacuoles (PSVs) were isolated from imbibed *L. albus* seeds following a protocol based on that described by Einhoff *et al.* (1986). The seeds were gently homogenized with chilled double-distilled water [5 ml $(g\ FW)^{-1}$ of tissue] and the homogenate filtered through cheesecloth and centrifuged at 350 *g* for 10 min. The pellet was discarded and the supernatant centrifuged at 17,500 *g* for 10 min. The resulting pelleted intermediate layer containing the PSVs was collected and resuspended in water to 5 ml g^{-1} of the initial seed FW. The crude PSV fraction was placed on to a 5% (w/v) Ficoll solution in water, which was layered on top of an equal volume of a 25% (w/v) Ficoll solution, and centrifuged at 500 *g* for 20 min. The isolated PSVs, forming a layer at the interface between the two different Ficoll concentrations, were removed with a micropipette. All operations were performed at 4°C. The PSVs were identified using a phase-contrast microscope (Leica DMRB, Wetzlar, Germany), after staining with 2% (w/v) potassium iodide in distilled water. From the mass of *Lupinus* cotyledons used as starting material, the purification yield and mass of purified PSVs, as well as the contribution of PSVs to the cotyledonary dry weight (DW) were calculated.

Measurement of enzyme activity

α-Mannosidase was used as a PSV marker and its activity was assayed essentially as described by Einhoff *et al.* (1986). A 0.005 M solution of *p*-nitrophenyl-α-D-mannopyranoside in 0.05 M sodium acetate buffer pH 5.0 was incubated with aliquots of enzyme preparations in a total volume of 150 μl for up to 15 min. The reaction was stopped by the addition of 0.2 M sodium carbonate buffer pH 9.0 (150 μl) and absorbance was measured at 405 nm in a Diagnostics Pasteur LP400 microplate reader (Sanofi, Marnes-la-Coquette, France). One enzyme unit (U) corresponds to the enzyme amount capable of releasing 1 μmol of 4-nitrophenol min^{-1}.

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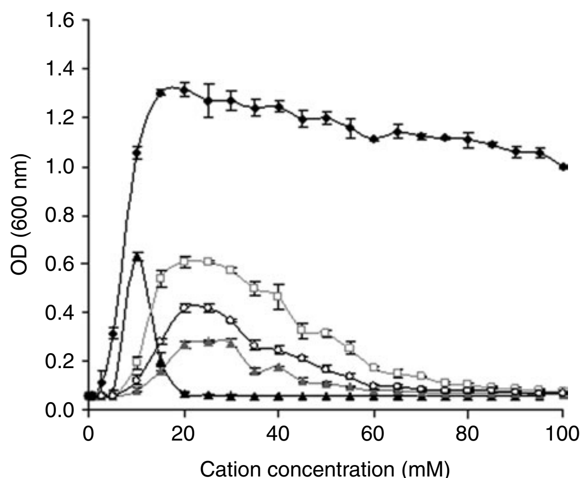

Figure 1. *Lupinus albus* globulin aggregation–disaggregation profiles when incubated in the presence of increasing concentrations (0–100 mM) of Be^{2+}(▲), Mg^{2+} (▲), Ca^{2+} (□), Sr^{2+}(○) and Ba^{2+}(◆) at pH 7.5. The values shown are the average of three determinations and the bars represent ± SD.

Turbidity measurements

The turbidity measurements of the globulins were made according to an adapted procedure based on the one described by Okubo *et al.* (1976): the globulin fraction obtained after desalting was quantified according to the Bradford method as modified by Ramagli (1999), and resolubilized in universal buffer (Britton and Robinson, 1931) diluted to one-third at pH 7.5, pH 6.5 or pH 5.5 for a final protein concentration of 2.2 mg ml^{-1}; 90 μl of the globulin solution was incubated with 10 μl of the different chloride salts (Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}) in concentrations ranging from 0 to 100 mM. Turbidity measurements were made spectrophotometrically at 600 nm in a PowerWave XS microplate reader (BioTek, Bad Friedrichshall, Germany) after an incubation period of 2 min; longer periods of incubation did not result in an increase in absorbance.

Alkaline earth element concentrations

Alkaline earth elements were extracted by digesting the dried residue of PSVs or of cotyledons in a 1:1 solution of 50% (v/v) HNO_3 and 50% (v/v) HCl, after which the samples were filtered and the volumes adjusted to 20 ml (PSVs) or 50 ml (cotyledons). Their concentrations were then determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) Ultima (Jobin-Yvon Horiba, Stanmore, Middlesex, UK). The wavelengths used were 313.042 nm for Be^{2+}, 285.213 nm for Mg^{2+}, 422.673 nm for Ca^{2+}, 346.446 nm for Sr^{2+} and 233.527 nm for Ba^{2+}. All plasticware and glassware used in PSV isolation were previously soaked in 50% (v/v) HCl to minimize mineral contamination. All measurements were made in triplicate. Blank assays yielded element concentrations that varied between 4 and 7% of those obtained for samples. Although this methodology may underestimate cation concentrations, due to their leakage from the organelles during purification, alternative approaches described in previous studies do not provide an accurate quantification. Among these are cryofixation and cryosectioning (Lott and Buttrose, 1978), which partially overcome cation leakage since the element distribution is not significantly affected by the sample preparation, but due to difficulties in standardizing the X-ray microanalytical technique, the measurements are only semi-quantitative or only allow a qualitative analysis of the radial distribution of elements (Bucking *et al.*, 2002).

Protein data search

A protein data search for *L. albus* conglutins was performed in the UniProt database [http://www.uniprot.org; (accessed 2010); Jain *et al.*, 2009]. The sequences chosen for β-conglutin (Q6EBC1), α-conglutin (Q53I54) and γ-conglutin (Q9FSH9 and Q9FEX1), were used for sequence homology searches in the Protein Data Bank [http://www.pdb.org; (accessed 2010); Berman *et al.*, 2000] using an expectation value, $E < 10^{-20}$.

Table 1. Alkaline earth cation content in cotyledons and protein storage vacuoles (PSVs) isolated from *Lupinus albus* seeds. Values are averages of three determinations ± SD

Alkaline earth cation	Cotyledons [μmol (g DW)$^{-1}$ of cotyledons]	PSVs (μmol in PSVs per g DW of cotyledons)
Be^{2+}	0.063 ± 0.042	ND
Mg^{2+}	43.6 ± 4.9	6.37 ± 0.95
Ca^{2+}	72.3 ± 2.5	43.9 ± 3.0
Sr^{2+}	0.0704 ± 0.0042	ND
Ba^{2+}	0.084 ± 0.021	ND

ND, not detected.

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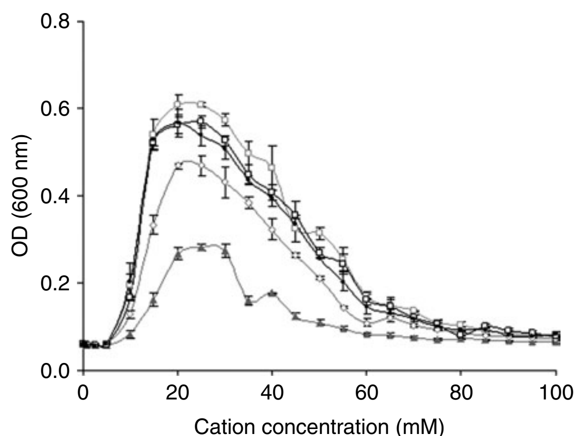

Figure 2. *Lupinus albus* globulin aggregation–disaggregation profiles when incubated in the presence of increasing concentrations (0–100 mM) of Mg^{2+} (▲), Ca^{2+} (□), or different combinations of Mg^{2+} and Ca^{2+}: 1:1 (◇), 1:7 (□) and 1:9 (●) at pH 7.5. Values shown are the average of three determinations and bars represent ± SD.

Results and discussion

On the basis of known cation electrostatic involvement in the macromolecular aggregation of legume seed storage proteins, a mechanism that ensures an efficient packing inside PSV, the alkaline earth cations were used to evaluate their effects not only on aggregation of *L. albus* globulins but also on disaggregation profiles.

Alkaline earth elements and in vitro *globulin self-aggregation–disaggregation*

To assess the ability that the alkaline earth elements have to promote *L. albus* globulin self-aggregation, turbidity measurements were used to follow its aggregation–disaggregation profiles. Purified globulins, at a time before hydrolysis was initiated (Ferreira *et al.*, 1995), were incubated in the presence of the various divalent cations: Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} (Fig. 1). Not all cations were equally effective in promoting globulin self-aggregation and disaggregation. For instance, Ba^{2+}, which caused the highest turbidity measurements, produced globulin aggregation at concentrations ranging from 5 to 20 mM. However, no globulin disaggregation was observed for higher Ba^{2+} concentrations. When compared with the other cations studied, Be^{2+} originated a distinct pattern of globulin aggregation–disaggregation; small changes in concentration in the range of 5–15 mM produced very high variations. Only Mg^{2+}, Ca^{2+} and Sr^{2+} produced similar globulin patterns of aggregation–disaggregation. Among these, the highest turbidity measurement was recorded for Ca^{2+} at 20 mM, followed by Sr^{2+} and Mg^{2+} at 25 mM. Cation-dependent formation of high-order aggregates of globulin molecules were previously observed by turbidity measurements and validated by isopycnic density gradient centrifugation (see figure 4 in Ferreira *et al.*, 1999). Above 65 mM no globulin aggregation could be detected using Mg^{2+}, Ca^{2+} or Sr^{2+}. Under the conditions studied, Ba^{2+} promoted an irreversible globulin aggregation, whereas the remaining cations generated a reversible globulin aggregation profile, with Be^{2+} producing a narrower dose-mediated reversibility than Mg^{2+}, Ca^{2+} or Sr^{2+}. This profile of reversible globulin aggregation for Mg^{2+} and Ca^{2+} was previously shown for *L. albus* globulins as well as for other legume seeds (figure 3 in Ferreira *et al.*, 1999) and was tentatively explained as an electrostatic process (Ferreira *et al.*, 2003).

Alkaline earth element concentrations inside PSVs

To determine which alkaline earth elements (Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}) can be effectively involved in the *in vivo* mobilization of legume storage proteins, their concentrations were measured in cotyledons and PSVs isolated from *L. albus* seeds (Table 1). As expected, Mg^{2+} and Ca^{2+} were by far the most abundant alkaline earth elements present, comprising 43.6 μmol and 72.3 μmol per g DW of cotyledons, respectively, whereas Be^{2+}, $Sr^{2+}$and Ba^{2+} were present in trace amounts. The cotyledonary contents of Mg^{2+} and Ca^{2+} are consistent with the values reported in the literature for the chemical composition of lupin seeds (Trugo *et al.*, 1993); however, for the minor components, Be^{2+}, Sr^{2+} and Ba^{2+}, there are no reports. In purified PSVs Mg^{2+} and Ca^{2+} were the only alkaline earth elements detected, 6.37 μmol and 43.9 μmol per g DW of cotyledons, respectively; the other elements were below the detection limit of the method

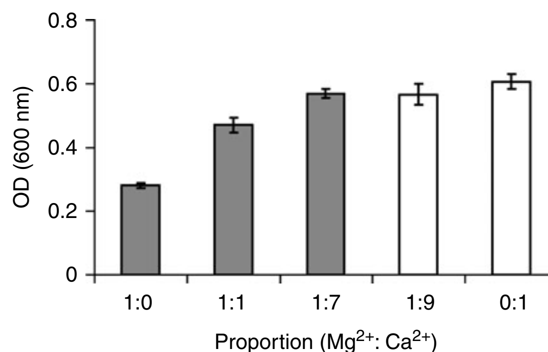

Figure 3. Maximum turbidity obtained for globulin aggregation in the presence of Mg^{2+}, Ca^{2+} and combined fractions of Mg^{2+} and Ca^{2+}(1:1, 1:7, 1:9). Columns represent the maximum turbidity obtained for 25 mM (grey columns) and 20 mM (white columns). Values shown are the average of three determinations and bars represent ± SD.

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Table 2. Proteins annotated in the Protein Data Bank (Berman *et al.*, 2000) that have sequence similarities, for an expectation value $E < 10^{-20}$, with β-(Q6EBC1), α-(Q53I54) and γ-(Q9FSH9 and Q9FEX1) conglutin sequences annotated in the UniProt database (Jain *et al.*, 2009) for *L. albus*. The ligands described to be at the surface of the proteins are indicated

Protein Data Bank ID (Accession no. : chains)	Protein description	Species	Score	Expectation value (E)	Described ligands at the protein surface
	β-conglutin	***Lupinus albus***			
1UIK:A,B,C	β-Conglycinin	*Glycine max*	444	1.0×10^{-124}	Mg^{2+}
1IPK:A,B,C	β-Conglycinin	*Glycine max*	432	1.0×10^{-121}	–
1IPJ:A,B,C	β-Conglycinin	*Glycine max*	432	1.0×10^{-121}	NAG
2EAA:A,B,C	7S Globulin-3	*Vigna angularis*	429	1.0×10^{-120}	CIT, Ca^{2+}
1UIJ:A,B,C,D,E,F	β-Conglycinin	*Glycine max*	429	1.0×10^{-120}	–
2EA7:A,B,C	7S Globulin-1	*Vigna angularis*	425	1.0×10^{-119}	Ca^{2+}
2CV6:A	8S α-Globulin	*Vigna radiata*	401	1.0×10^{-112}	–
2CAV:A; 2CAU:A	Canavalin	*Canavalia ensiformis*	393	1.0×10^{-109}	–
2PHL:A,B,C	Phaseolin	*Phaseolus vulgaris*	283	2.0×10^{-76}	NAG, PO_4^{3-}
1PHS:A	Phaseolin	*Phaseolus vulgaris*	283	2.0×10^{-76}	–
1CAX:B,D,F; 1CAW:B; 1CAV:B; 1CAU:B	Canavalin	*Canavalia ensiformis*	194	2.0×10^{-49}	–
1CAX:A,C,E; 1CAW:A; 1CAV:A; 1CAU:A	Canavalin	*Canavalia ensiformis*	188	1.0×10^{-47}	–
1DGW:A; 1DGR:A,B,C	Canavalin	*Canavalia ensiformis*	186	5.0×10^{-47}	–
1DGW:Y; 1DGR:M,W,Y	Canavalin	*Canavalia ensiformis*	119	8.0×10^{-27}	–
	α-conglutin	***Lupinus albus***			
3KSC:A,B,C,D,E,F	Prolegumin	*Pisum sativum*	327	9.0×10^{-90}	GOL, SO_4^{2-}
1FXZ:A,B,C	Proglycinin	*Glycine max*	318	7.0×10^{-87}	–
1UD1:A,B,C	Proglycinin	*Glycine max*	314	9.0×10^{-86}	–
1UCX:A,B,C	Proglycinin	*Glycine max*	313	2.0×10^{-85}	–
3C3 V:A	Arachin	*Arachis hypogaea*	272	4.0×10^{-73}	–
1OD5:A,B; 2D5H:A,B,C,D,E,F	Glycinin	*Glycine max*	219	3.0×10^{-57}	–
2D5F:A,B	Proglycinin	*Glycine max*	219	3.0×10^{-57}	–
2EVX:A	Globulin	*Cucurbita maxima*	186	4.0×10^{-47}	–
2E9Q:A	Globulin	*Cucurbita maxima*	186	4.0×10^{-47}	PO_4^{3-}
3FZ3:A,B,C,D,E,F	Amandin	*Prunus dulcis*	162	4.0×10^{-40}	Na^{+}, Ca^{2+}
3EHK:A,B,C,D,E,F	Amandin	*Prunus dulcis*	162	4.0×10^{-40}	–
3KGL:A,B,C,D,E,F	Globulin	*Brassica napus*	158	8.0×10^{-39}	SO_4^{2-}
	γ-conglutin	***Lupinus albus***			
3HD8:A,C	Xylanase inhibitor-IIA	*Triticum aestivum*	131/132	7.0×10^{-32}, 4.0×10^{-31}	–
1T6G:A,B; 1T6E:X	Xylanase inhibitor-I	*Triticum aestivum*	128/131	7.0×10^{-30}, 1.0×10^{-30}	GOL
2B42:A	Xylanase inhibitor-I	*Triticum aestivum*	114/116	1.0×10^{-25}, 4.0×10^{-26}	–

NAG, *N*-Acetyl-D-glucosaminidase; CIT, citric acid; GOL, glycerol.

employed, and thus too little to participate in globulin mobilization. Because PSVs represent approximately 52% of *L. albus* cotyledonary DW (0.519 ± 0.034 g DW of PSVs per g DW of cotyledons), it is calculated that 14.6% and 60.7% of the seed Mg^{2+} and Ca^{2+}, respectively, are located within PSVs. Considering that the Ca^{2+} concentration is approximately seven times higher than that of Mg^{2+} in PSVs, and almost twice that of Mg^{2+} in cotyledons (Table 1), the patterns of globulin aggregation–disaggregation were determined *in vitro* for different combinations of cation concentrations: 1:1, 1:7 and 1:9 (Fig. 2).

The combined concentrations of Mg^{2+} and Ca^{2+} resulted in globulin aggregation–disaggregation profiles intermediate between the globulin profiles obtained for Mg^{2+} and Ca^{2+} alone, with the turbidity increasing as Ca^{2+} proportion increased. For each condition, the total cation concentration that is able to promote maximum globulin aggregation decreases as the Ca^{2+} contribution in the fractions increases (Fig. 3). The requirement of less Ca^{2+} than Mg^{2+} to promote higher turbidity indicates that these two cations may have distinct contributions to globulin aggregation and disaggregation, with the former being the most efficient.

Proposed dual role for Ca^{2+} and Mg^{2+} from a structural standpoint

According to the globulin aggregation–disaggregation profile observed, both Ca^{2+} and Mg^{2+} are expected to be involved *in vivo* in globulin structural changes. A hypothesis is formulated which underlies their proposed role in the way storage globulins are efficiently packed during legume seed formation, free from the action of neighbouring proteases, and subsequently dismantled and subjected to proteolytic digestion during seed germination and seedling growth. This relates to the *in vitro* response of the globulins to Ca^{2+} or Mg^{2+} and the amount of these cations inside PSVs. The annotated protein structures were scrutinized to verify if either Ca^{2+} or Mg^{2+} promote crystallization, by aiding the interaction between these protein molecules. Although crystallization is not aggregation, but rather a salting out of protein molecules in an ordered and repetitive manner, it is still possible to infer the position of Ca^{2+} and Mg^{2+} in the protein structure.

The sequences annotated in the UniProt database (Jain *et al.*, 2009) for β-conglutin (Q6EBC1), α-conglutin (Q53I54) and γ-conglutin (Q9FSH9 and Q9FEX1) from

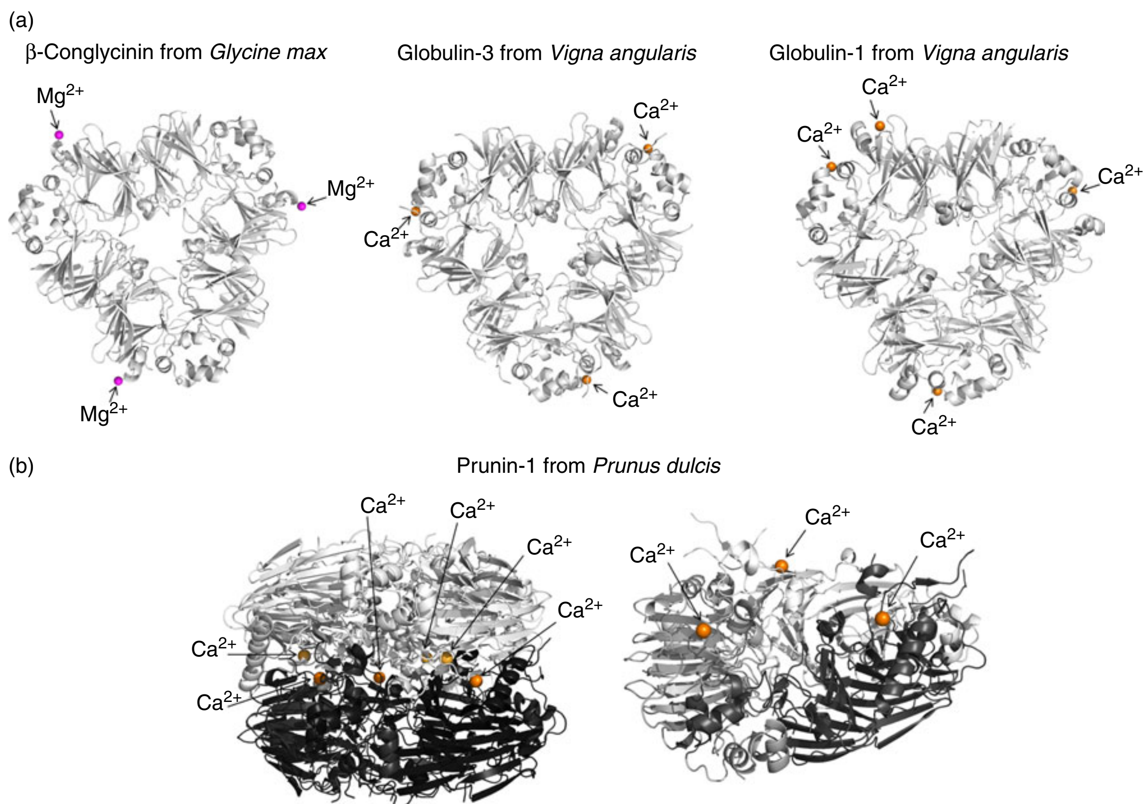

Figure 4. Three-dimensional structures representative of β-conglutin homologues (a) and α-conglutin homologues (b), with Mg^{2+} represented by pink spheres and Ca^{2+} by yellow spheres (see online at http://www.journals.cambridge.org/ssr for a colour version of this figure). The precise location of the spheres is highlighted by arrows. The structures presented were prepared using Pymol (DeLano, 2002).

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L. albus were chosen for sequence homology searches in the Protein Data Bank (Berman *et al.*, 2000). Several hits that are mainly from other legume crops were observed for all three proteins (Table 2). Some were reported for β- and α-conglutins that showed the presence of either Ca^{2+} or Mg^{2+} in their crystal structure. Only three of the β-conglutin homologues showed the presence of Ca^{2+} or Mg^{2+}, namely β-conglycinin from *Glycine max* (1UIK) which is reported to contain Mg^{2+} (Maruyama *et al.*, 2004), and globulin-3 and globulin-1 from *Vigna angularis* (2EAA and 2EA7, respectively) which are reported to contain Ca^{2+} (Fukuda *et al.*, 2008) (Fig. 4a). These elements are positioned at the protein surface and are co-ordinated by protein residues and water molecules or by just water molecules, as observed for β-conglycinin from *Glycine max*. For α-conglutin (Q53I54), there is a single homologous protein, prunin-1 from *Prunus dulcis* (3FZ3), where the presence of Ca^{2+} is reported (Jin *et al.*, 2009). In this case, Ca^{2+} is at the surface of each subunit, allowing the transition between two trimers in the hexameric structure of the native protein (Fig. 4b). From all the hits reported for γ-conglutin, none shows the presence of either Ca^{2+} or Mg^{2+}, in agreement with the reduced aggregation reported for γ-conglutin in the presence of Ca^{2+} or Mg^{2+}; this protein preferentially interacts with Zn^{2+} (Ferreira *et al.*, 1999, Duranti *et al.*, 2001).

β-Conglutin was previously reported to have the highest total globulin fraction aggregation, not only because it is the most abundant lupin storage protein, but also because a higher turbidity was observed in the presence of Mg^{2+} and Ca^{2+}(1:1) when compared to α-conglutin (Ferreira *et al.*, 1999). Despite the reported lectin-like properties exhibited by β-conglutin (Sharon and Lis, 1990), its self-aggregation was previously proposed to be electrostatic rather than lectin-mediated (Ferreira *et al.*, 2003). Nevertheless, either mechanism requires Ca^{2+} and Mg^{2+} to promote β-globulin self-aggregation. The fact that for the same amount of protein, β-conglutin produces higher turbidity measurements than α-conglutin (see figure 1 in Ferreira *et al.*, 1999), suggests that β-conglutin is more prone to bind Ca^{2+} and/or Mg^{2+}.

Proposed dual physiological role of Ca^{2+} and Mg^{2+}

PSVs are acidified following seed germination (Otegui *et al.*, 2006; He *et al.*, 2007). Given the electrostatic nature of the globulin supramolecular aggregates, a decrease in pH will alter the net charge of the protein aggregates, influencing globulin interaction with Ca^{2+} and Mg^{2+}; however, globulin aggregation–disaggregation profiles were screened at a neutral pH (Ferreira *et al.*, 1999, 2003). To assess the alterations induced by a mildly acidic pH, the globulin aggregation–disaggregation profiles were evaluated at pH 6.5 and pH 5.5 in the presence of Mg^{2+} (Fig. 5a), Ca^{2+} (Fig. 5b) and also Ca^{2+} and Mg^{2+} combined (1:1, 1:7, 1:9) (data not shown). The values obtained were compared with those achieved at pH 7.5. The globulin profile differs with pH, with the maximum values for turbidity gradually increasing and shifting towards lower Ca^{2+} and Mg^{2+} concentrations as the pH drops from 7.5, through 6.5, to 5.5. A similar behaviour was observed for the combined Mg^{2+} and Ca^{2+}(1:1, 1:7, 1:9) fractions (data not shown), once again with profiles that are intermediate between those of Mg^{2+} and Ca^{2+} alone. This decrease in globulin solubility due to acidification is in accordance with the previous observation that a decrease in pH (5.5–6.0) promotes an increase in the aggregation of legumin-type globulins from *Arabidopsis thaliana* (Gruis *et al.*, 2004).

A decrease in pH will exert two effects on legume globulins: (1) as it gradually approaches globulin pI values, which are mainly acidic (Crouch and Sussex, 1981; García *et al.*, 1997; Magni *et al.*, 2007), electrostatic interaction changes will decrease protein solubility to

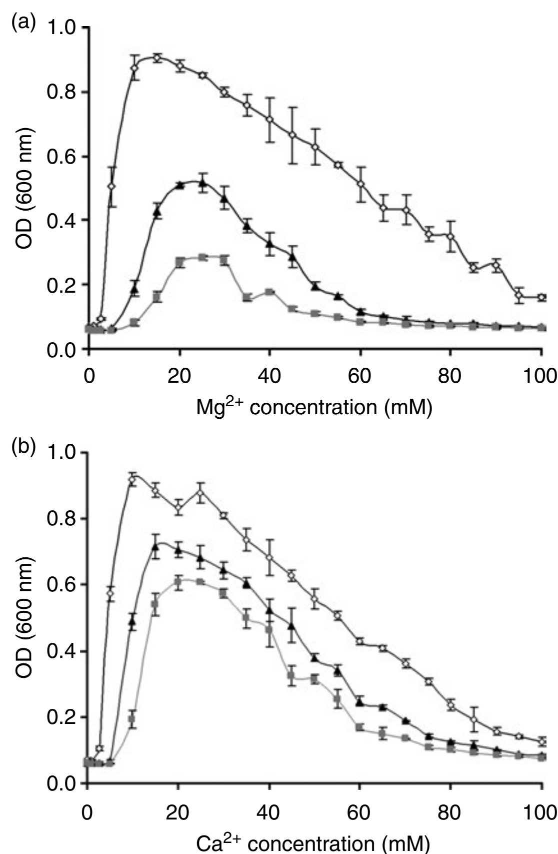

Figure 5. *Lupinus albus* globulin aggregation–disaggregation profiles obtained *in vitro* at different pH values. The isolated globulins were incubated in the presence of increasing concentrations (0–100 mM) of (a) Mg^{2+} and (b) Ca^{2+} at pH 7.5 (■), pH 6.5 (▲) or pH 5.5 (◇). Values shown are the average of three determinations and bars represent ± SD.

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a minimum value; (2) as the number of negative charges on the globulin surface is reduced, so will the Ca^{2+} and/or Mg^{2+} binding sites, shifting the equivalence point of maximum turbidity to lower cation concentrations. The slightly different globulin aggregation–disaggregation profile observed for Ca^{2+} at pH 5.5, when compared either with pH 6.5 or pH 7.5 profiles (Fig. 5b), may result from these different contributions by causing altered conglutin interactions with this cation (figure 1 in Ferreira *et al.*, 1999). The data in Fig. 5 can have physiological significance, following germination, if an increase in free Ca^{2+} and Mg^{2+} concentrations is concomitant with a drop in pH inside the PSVs.

In addition to insoluble protein deposits and soluble storage proteins, PSVs also contain a matrix of globoids of phytic acid and oxalate crystals (Jiang *et al.*, 2000). Phytate, a fully phosphorylated form of inositol, chelates most metal ions, such as Mg^{2+} and Ca^{2+} which, in addition to their participation in crystal oxalate formation (mainly calcium oxalate), makes the active participation of these two cations in metabolic processes unlikely (Rendle, 1888; Clarkson, 1980; Ilarslan *et al.*, 2001; Franceschi and Nakata, 2005). This PSV storage function is in agreement with the high amounts of Ca^{2+}, and to a lesser extent Mg^{2+}, detected inside *L. albus* PSVs (Table 1).

Upon seed germination and seedling growth, the low free cation content can be reversed by the combined action of several events that arise from PSV acidification, namely phytase activation and oxalate crystal dissolution (Franceschi, 1989). In *L. albus*, phytase has an optimal pH of 5.0 (Greiner, 2002) and a decrease in phytate content, and also oxalate crystal dissolution, will cause a substantial increase in the free cation content inside PSVs. In lupin, phytase maximum activity was reached 4 d after seed imbibition (Greiner, 2002), which is coincident with the beginning of globulin degradation (Ferreira *et al.*, 1995). Acidification inside PSVs also mediates the activation of several other enzymes, such as oxalate oxidase, an enzyme proposed to participate in the degradation of the oxalate liberated from the crystals (Dumas *et al.*, 1995, Kanauchi *et al.*, 2009), and subtilisin-like serine proteases, proposed to be involved in storage protein mobilization in soybean seeds (He *et al.*, 2007). Furthermore, a protease from *Vigna radiata* seeds involved in storage protein mobilization was recently reported to be activated by Ca^{2+} (Khan *et al.*, 2010). These physiological events can be related to the *in vitro* profile observed for globulin aggregation and disaggregation with decreasing pH and in the presence of increasing Mg^{2+} and Ca^{2+} concentrations (Fig. 6).

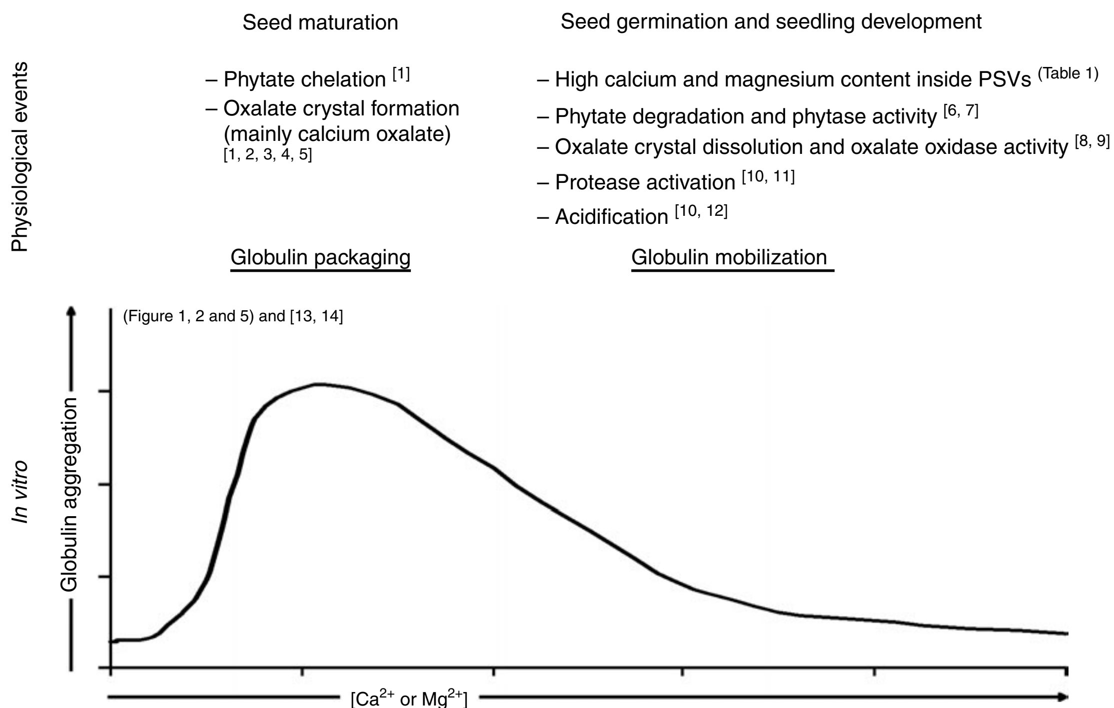

Figure 6. Schematic representation of the main physiological events that occur during seed maturation, seed germination and seedling growth, correlated with *in vitro* globulin aggregation and disaggregation profile in the presence of increasing free Mg^{2+} and Ca^{2+} concentrations. References: [1]Jiang *et al.*, 2000; [2]Clarkson, 1980; [3]Franceschi and Nakata, 2005; [4]Ilarslan *et al.*, 2001; [5]Rendle, 1888; [6]Franceschi, 1989; [7]Greiner, 2002; [8]Dumas *et al.*, 1995; [9]Kanauchi *et al.*, 2009; [10]He *et al.*, 2007; [11]Khan *et al.*, 2010; [12]Otegui *et al.*, 2006; [13]Ferreira *et al.*, 1999; [14]Ferreira *et al.*, 2003.

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Although there is no doubt that dry seed PSVs contain proteases, their mechanisms of action in storage protein mobilization remains unclear. Our results suggest that relatively low free Mg^{2+} and Ca^{2+} concentrations that occur during seed maturation contribute to globulin aggregation by promoting protein–divalent cation–protein interactions, thus explaining the tight storage protein packaging inside PSVs. An increase in free cation concentration, during and following seed germination, could contribute to globulin disaggregation by oversaturation of the protein–divalent cation binding sites, causing the positively charged globulin molecules to repel each other (Ferreira *et al.*, 2003). These globulin aggregation events could modulate PSV protease accessibility to their substrates. Globulin aggregation could block the access of proteases to the substrate, whereas aggregate dismantling could contribute to protease accessibility, allowing globulin mobilization. To further investigate this, the environment inside PSVs has to be fully characterized.

In conclusion, the coexistence of storage proteins with proteases responsible for their degradation is well documented, although globulin packaging and mobilization inside legume PSVs is still poorly understood. By integrating the *in vitro* data for globulin reversible aggregation in the presence of Ca^{2+} and Mg^{2+} with the physiological events that occur during legume seed development, we suggest that the low free Ca^{2+} content inside PSVs, and to a lesser extent Mg^{2+}, can mediate globulin packaging and therefore contribute to protection against proteolytic attack. Following seed germination and seedling growth, an increase in free cation levels could reverse the process by promoting globulin disaggregation and protease accessibility to the substrate.

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